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Award Number: DAMD17-96-1-6322

TITLE: Immunity to HER-1/neu Protein

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REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | October 1999 | Annual (23 Sep 98 - 22 Sep 99) |

**4. TITLE AND SUBTITLE**
Immunity to HER-1/neu Protein

**5. FUNDING NUMBERS**
DAMD17-96-1-6322

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**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** ***(Maximum 200 Words)***

The HER-2/neu (HER2) proto-oncogene is amplified and overexpressed in 20-40% of invasive breast cancers. HER2 over-expression is associated with aggressive disease and is an independent predictor of poor prognosis in several subsets of patients. HER2 may also be related to cancer formation, with overexpression detectable in 50-60% of ductal carcinomas *in situ* (DCIS).The overall goal for the proposal is to develop the knowledge base necessary to develop vaccine and T cell therapy strategies directed against HER2. Preliminary studies discovered that some patients with breast cancer have existent CD4+ helper T cell immunity and antibody-mediated immunity to HER2. HER2 is a self protein. Before our studies it was assumed patients would be immunologically tolerant to HER2 and that immunity could not be generated. Our prior studies demonstrated that immunity to HER2 is induced in some individuals by virtue of the presence of growing cancer expressing the antigen and gives credence to the concept that HER2-specific immunity can potentially be used in therapy without destroying normal tissue. This grant is exploring issues important for developing HER2 specific vaccines and T cell therapy. In addition, the demonstration of immunity to HER2 offers the opportunity to explore host-tumor interactions in a well-defined antigen system.

**14. SUBJECT TERMS**
Immunity, Vaccine, T cells, Antibodies, T cell Therapy, Dendritic Cells

**15. NUMBER OF PAGES**
20

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

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N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

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Mary L. O... 11/17/99

PI - Signature Date

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## INTRODUCTION:

The HER2 (HER2) proto-oncogene is amplified and overexpressed in 20-40% of invasive breast cancers. HER2 over-expression is associated with aggressive disease and is an independent predictor of poor prognosis in several subsets of patients. HER2 may also be related to cancer formation, with overexpression being detectable in 50-60% of ductal carcinomas *in situ* (DCIS). The overall goal for the proposal is to develop the knowledge base necessary to develop vaccine and T cell therapy strategies directed against HER2. Preliminary studies prior to the grant discovered that some patients with breast cancer have existent CD4+ helper T cell immunity and antibody-mediated immunity to HER2. HER2 is a self-protein. Therefore, before our studies, it had been assumed that patients would be immunologically tolerant to HER2 and that immunity could not be generated. Our prior studies demonstrating that immunity is already present in some patients with breast cancer implied that immunity to HER2 is induced in some individuals by virtue of the presence of growing cancer expressing the antigen and gives credence to the concept that HER2-specific immunity can potentially be used in therapy without destroying normal tissue. The current grant is exploring issues important for developing HER2 specific vaccines and T cell therapy. In addition, the demonstration of immunity to HER2 offers the opportunity to explore host-tumor interactions in a well-defined antigen system. During this funding period we have begun to immunize patients against the HER2 proteins in terms of peptide-based vaccines. These studies have led to easier expansion of CD4+ and CD8+ T cells specific for HER2. In addition, we have begun to utilize animal models in the form of neu transgenic mice (neu-tg) to begin to address some of the issues of the functionality of the response. Thus, work accomplished in this grant has already led to the development of a first generation peptide based vaccine targeting HER2. Ongoing vaccine studies with HER2 peptides have shown that immunity to HER2 can be elicited and augmented by vaccination of human with HER2+ cancers. Three problems remain. (1) Not all patients respond, (2) Ab responses are weak, (3) CTL responses are HLA-A2 restricted with a peptide vaccine. This progress report details not only the progress on the original aims defined, but also new studies undertaken in line with the original grant goals, to develop approaches to overcome the above problems.

**Specific Aim #1 is examining Ab immunity to HER2.** Preliminary data showed that Ab immunity to HER2 could be detected in the sera of some patients with breast cancer. Studies were proposed to determine the frequency of Ab immunity, the relative frequency of functional Ab and to determine whether responses to HER2 are beneficial or detrimental. Additional studies were proposed to determine whether immunity to HER2 can serve as a marker for early cancer and/or whether changes in level represent a marker for relapse.

**Specific Aim # 2 is examining CD4+ T cell immunity to HER2**. Preliminary data showed that some patients with HER2-positive breast cancers exhibit primed CD4+ helper T cell responses to HER2. Finding existent T cell immunity is encouraging for the eventual use of T cell vaccines and T cell therapy. In animal models CD4+ T cells can be effective against abundant soluble proteins. The extracellular domain (ECD) of HER2 is shed abundantly in some patients. Studies were proposed to determine the prevalence of CD4+ responses in patients with HER2+ tumors and to determine whether changes in immunity occur with therapy and relapse. One limitation to the development of human anti-cancer vaccines and T cell therapy is that determination of immunogenicity conventionally requires immunization *in vivo*. Therefore, much effort was proposed to develop methods of priming *in vitro*. Preliminary data in the grant proposal described prior studies developing a culture system using dendritic antigen presenting cells that allows exceedingly rapid priming *in vitro*. Studies were proposed to determine whether the priming system is reproducible enough and powerful enough to allow determination of which peptides are immunogenic and which peptide specific T cells are capable of responding to whole HER2 protein. Identification of epitopes is important for eventual incorporation into peptide-based vaccines or for use to stimulate T cells *in vitro* for T cell therapy regimens.

**Specific Aim # 3 is examining CD8+ CTL immunity to HER2**. Preliminary data for the grant showed that CD8+ CTL could be primed to HER2 peptides *in vitro* and that primed peptide-specific CTL can lyse HER2-positive cancer cells. However, the systems employed are extremely fastidious. *In vitro* priming was to be developed and used to identify the immunogenic epitopes of HER2. Additional studies were proposed to determine whether patients with breast cancer have existent CTL immunity to HER2, as had previously been described for patients with ovarian cancer. For patients with CTL immunity and HER2-positive cancers, studies were proposed to determine the prevalence of CD8+ responses in patients with HER2+ tumors and the evolution of immunity with therapy and relapse. Finally, studies were proposed to determine whether HER2-specific CD8+ CTL derived from patients with breast cancer can lyse autologous tumor and can be expanded *in vitro* to the extent needed for adoptive therapy.

**BODY:**

The BODY of the Progress Report will be organized according to the STATEMENT OF WORK in the original grant proposal.

**Specific Aim #1: To examine Ab immunity to HER2.**

Task 1: Months 1-48 To determine the frequency of Ab, the biologic function of HER2 specific Ab, Ab correlation with HER2 overexpression and Ab correlation with circulating ECD. Previous progress reports indicated the incidence of HER2 antibody is approximately 20% in HER2-positive breast cancer patients and are found in approximately 11% of breast cancer patients in general (HER2 status unknown) (1). In addition, the presence eof HER2 specific antibodies has been documented in other HER2 overexpressing cancers including colon cancer (2) and prostate cancer (3). We have just completed a final study concentrating on the HER2 specific antibody immune response in advanced stage breast cancer patients with HER2- positive tumors. These patients were first tested for their ability to mount an immune response by recall antigen skin testing. None of the patients were anergic. Of 45 patients studied, 8% had evidence of a pre-existent antibody response to the HER2 protein. The antibodies mapped equally to the ECD and ICD. Thus, in the studies accomplished to date we have determined the frequency of detecting HER2 antibodies in patients with HER2 overexpressing cancers is low- in a minority of patients, maximum of 20% in early stage and 10% in more advanced stage and that the presence of antibodies correlate significantly with the presence of HER2 overexpression in the tumor(2, 4). There has been no correlation between level of antibody with level of circulating ECD in these studies most likely due to the fact that few patients have circulating ECD and antibodies. Thus, this task has been completed. Current studies are utilizing sera that contain antibodies to HER2 to screen E. coli libraries to probe for novel antigens using a technique called SEREX. Data in the last progress report demonstrate the feasibility of the procedure and new data presented here demonstrates the identification of several potential novel breast cancer antigens defined by this method.

**Table 1.** Identity of five cDNA clones from a primary SEREX screen of the breast cancer cell line ZR-75.1 using serum from a breast cancer patient.

| CLONE | EST (PROTIEN) |
|---|---|
| 1 | No match |
| 2 | dbj/C15189 |
| 3 | gb/AA460610 (TNF Receptor) |
| 4 | gb/AA233267 |
| 5 | dbj/C15189 |

Task 2: Months 1-24 To examine biologic function of Ab binding to the ECD. Few patients with endogenous antibodies to ECD have been identified to yield substrate for these studies. Work in previous progress reports identified an epitope from HER2 p328 that has implicated as a site for potential antibody interference with growth factor receptor function. Thus peptide epitope has been incorporated into a vaccine and patients are actively being immunized with the peptide. We anticipate within the next year of funding we will be able to discern functional effects on antibody responses in patients boosted with the vaccine. Until then, we have turned to an animal model to help explore the role of an endogenous antibody response on the growth of HER2 overexpressing tumors *in vivo*.

Studies examined the use of ECD protein to elicit Ab capable of suppressing the growth of HER+ cancers. Preliminary experiments examined serum from mice immunized to ECD expressed by mouse L cells. Antibody from mice immunized to ECD could suppress proliferation of SKBR3, but not MCF7. SKBR3 is a breast cancer line that overexpresses HER2. MCF7 does not overexpress HER2. A variety of different adjuvants have been tested with IFA yielding the highest titers (Table 2). Additional adjuvants are being tested.

**Table 2.** Anti-ECD antibody titers in mice immunized twice with L cell derived ECD

| Immunization | Anti-ECD Antibody Titer |
|---|---|
| ECD/IFA | $1 \times 10^6$ |
| ECD/Montanide | $2 \times 10^6$ |
| ECD/Leif | $6.4 \times 10^4$ |
| ECD/alum | $2.5 \times 10^5$ |

In rats immunization to the entire ECD was less immunogenic than immunization with peptides. Also many potential epitopes are contained in the ICD. Thus, fusion proteins containing portions of the ECD and ICD are being

constructed as potential vaccine immunogens. Protein expressed by different systems can have different properties as vaccines due to difference in post-translational modifications. Also, protein made by two different systems are needed to evaluate vaccine regimens to make sure the responses to contaminants are not being measured and confused with responses to the nominated HER2 protein. We have thus chosen to develop the Bac-To-Bac baculovirus expression systems (GibcoBRL) in High Five Cells. This system allows for rapid protein expression. Once the system is worked out it can be applied to expression of HER2 proteins quickly. At present HER2 fusion proteins have been cloned into baculovirus and shown to express protein (Figure 1).

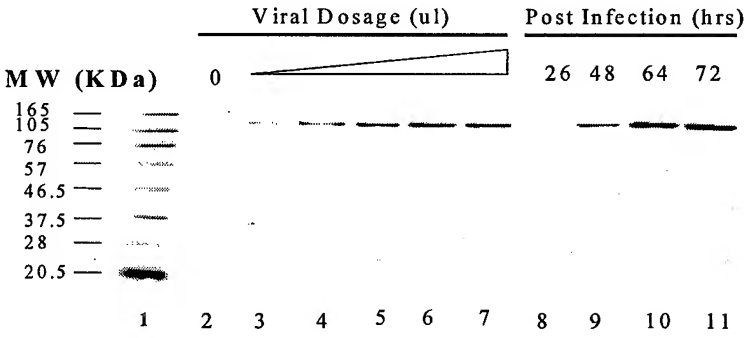



**Figure 1.** Expression of ECD fusion proteins in insect cells by recombinant baculovirus BV/HER2-fusion protein. One half million High 5 cells in 6-well plate were infected with different dosages of the recombinant virus (lane 2 – 7), or infected for different periods of time with the same dosage of virus (lane 8 – 11). Supernatants of the infected samples were collected, run on SDS-PAGE gel under reducing conditions and evaluated by Western blot with a monoclonal antibody against HER2 (c-neu Ab3, CalBiochem, USA). Lane 1, biotinylated protein marker; lane 2 to lane 7, supernatants collected 48 hours post-infection with 0, 8, 16, 42, 84 and 170 ul of BV/HER2 fusion ($3X10^5$ Pfu/ml); lane 8 –11, supernatants collected at 26, 48, 64 and 72 hours post-infection from the cells infected by 42 ul of the virus stock.

Task 3: Months 1-48 To determine whether responses to HER2 are beneficial or detrimental, stratifying for function. These studies are ongoing and actively accruing patients. The studies require sera from large cohorts of breast cancer patients. The HER2 status needs to be known for all and all need to be treated with the same regimen. A major problem in breast cancer research in general and our work specifically, is a lack of sera from breast cancer patients linked to evaluable clinical databases. Large volume, well-defined, breast cancer clinical databases with long-term follow up and corresponding serum samples are essential for answering many important questions about breast cancer etiology, prevention, diagnosis, and treatment and are sorely lacking at this time.

Therefore, to answer the questions in Aim #1 Task #3 we set up a serum bank for the National Surgical Adjuvant Breast and Bowel Project (NSABP). We began collecting sera as of the date of 3/1/97. The NSABP maintains a comprehensive clinical database on study participants, but has not previously collected sera, with the exception of their cancer prevention trial called P-01. Funds from the current grant are not going to fund the serum bank, but will be used to ask specific questions posed on the grant as to the role of HER2 Ab on outcome. In addition, with the NSABP, we have initiated a study specifically designed to determine whether responses to HER2 are beneficial or detrimental. A study of antibody responses to HER2 will be performed in the context of NSABP B-27. Briefly, protocol B-27 is designed to determine whether 4 cycles of pre-operative or post-operative Taxotere given after 4 cycles of pre-operative Adriamycin (A) and cyclophosphamide (C) will more effectively prolong disease-free survival and overall survival than 4 cycles of pre-operative AC alone. Protocol B-27.1 has been designed to obtain and analyze serum from B-27 patients for the presence of HER2 circulating ECD and antibody and to correlate these factors with tumor response to pre-operative chemotherapy and survival.

Protocol B-27.1 requires patient blood to be drawn by the NSABP pre-therapy, post neo-adjuvant chemotherapy, post surgery, at 12 month follow up and at the first relapse. The sera is being processed and stored for evaluation of HER2 antibody. Both fresh frozen and paraffin-embedded tissue specimens are being collected and stored at the NSABP headquarters. The NSABP database includes information on demographics, risk factors, family history, clinical and

pathologic factors, characteristics of tumor, treatment and outcome. Sera collection continues at the time of this progress report. Serial serum is beginning to be collected on the same individuals. All patients are receiving the same adjuvant chemotherapy regimen. Thus, the variable of HER2 reactivity can be analyzed. Stored sera will be examined in batches for Ab to whole HER2 protein, ICD and ECD. The function of ECD reactive Ab will be determined. Circulating serum ECD levels will be measured. The level of HER2 overexpression on primary tumor will be determined by immunocytochemistry on tissue blocks by NSABP reference pathologists. The NSABP statistical group will provide correlations.

Task 4: Months 1-48 To determine whether Ab varies predictably with therapy and recurrence. Levels of antibody might correlate with important clinical parameters. Thus, following levels of Ab might provide information useful for decision making. When tumor progresses or relapses, the increase in HER2 antigen load might be expected to stimulate rising titers. A rise in Ab titer might serve as a harbinger of relapse. While the study described in Task 3, NSABP 27.1, will give prognostic information concerning the correlation of HER2 specific antibody response and survival, sufficient time points of sera will be collected that we may be able to discern whether HER2 antibody levels vary predictably with recurrence. The patients included in the study above will have HER2 protein overexpression assessed on their primary tumor as well as circulating levels of ECD, shed HER2 protein, measured. Therefore, antibody data collected on these patients will allow the comparison of rise in antibody titer to the measurement of a more "classic" serum tumor marker; shed ECD protein.

We have initiated collaboration with Dr. Saul Rivkin at the Tumor Institute at Swedish Hospital here in Seattle. Dr. Rivkin's group is responsible for the primary treatment and management of over 50 newly diagnosed breast cancer patients/year. In this study, we propose to measure the antibody levels against HER2 in patients with HER2-positive breast cancer, stages I-IV, at different times during their disease course. All new patients with breast cancer, seen at the Tumor Institute over the next 12 months, will be approached to participate. Antibody levels would be drawn at diagnosis and followed at 3 month intervals throughout the patient's treatment and follow-up. Correlation between HER2-specific antibody levels, treatment responses, disease progression, and prognosis will be assessed. The estimate time of follow-up is 3 years.

Task 5: Months 13-48 To determine whether immunity to HER2: (a) is present in patients with DCIS; (b) correlates with progression of HER2+ DCIS to HER2 negative invasive cancer; and (c) represents a possible marker for early cancer. Studies of breast cancer biopsies show that HER2 levels are increased in the majority of DCIS specimens, but are not seen in atypia or dysplasia. Thus, overexpression of HER2 appears to be associated with malignant transformation and early neoplasia, but not benign proliferative diseases of the breast. This observation raises the question whether HER2-negative invasive ductal breast cancer arises from HER2 positive DCIS and whether HER2 immunity plays any role in immunoselection of progressive HER2 negative invasive ductal cancer from HER2-positive DCIS. Evaluating the immune response of newly diagnosed patients with DCIS and comparing that response to newly diagnosed patients with invasive breast cancer would lead to a better understanding of the interaction between the immune system and HER2 positive cancer.

To date, two new collaborations have been formed to collect material specifically on patients with DCIS. We started collaboration with Dr. Carol J. Fabian at the University of Kansas Medical Center. Dr. Fabian is conducting a Phase I study of a new selective estrogen receptor modulator (SERM) through the Chemoprevention Branch of the NCI. Patients with DCIS, T1, and T2 breast cancer will receive the agent for the interval between initial diagnostic biopsy and subsequent definitive surgical resection. HER2 antibody levels will be assessed on these patients as well as HER2 overexpression in DCIS or invasive breast cancer. In addition, the group will collect sera on all patients with DCIS evaluated, even those who do not enter the study. It is hoped collecting samples within the context of a Phase I treatment study will increase the population available for sera collection. Secondly, we have started a study with the mammography tumor registry here at the FHCRC with Dr. Nicole Urban. Patients with abnormal mammogram and undergoing biopsy will have blood drawn at the time of biopsy. We will evaluate the level of HER2 antibody, as well as p53 antibody and c-myc antibody, in the blood and correlate the presence of an immune response to these oncogenic proteins with abnormal pathology.

Finally, sera from NSABP P-01 protocol provides a unique resource to address these issues. Protocol P-01 is designed to test the hypothesis that long-term treatment with tamoxifen is effective in preventing invasive breast cancer. Serum is being collected on 16,000 individuals. It is projected that 325 of the subjects will develop breast cancer over the next 8 years. By examining serial sera for Ab at the time of diagnosis of malignancy, at fixed future time points and at the time of entry onto the protocol it should be possible to determine whether immunity predates diagnosis. The prevalence of Ab to HER2 will be too low to provide a general screening assay for breast cancer.

Task 6: Months 1-48 To determine whether immunity to HER2 correlates with outcome of 2B1 bispecific Ab therapy. TASK COMPLETED AS OF LAST PROGRESS REPORT.

**Specific Aim # 2: To examine CD4+ T cell immunity to HER2.**

Task 7: Months 1-36 To develop *in vitro* priming with dendritic APC to generate HER2-specific CD4+ T cells and to identify the epitopes recognized. As part of continued immunological efforts to validate the ICD of HER2 as a vaccine candidate, an *in vitro* priming protocol involving recombinant Adenovirus infection of DC was developed. An Adenovirus vector deleted for E1A and recombinant for the ICD was constructed and used to infect DC. Following maturation of the DC with CD40-L, priming cultures were initiated that contained $1.3 \times 10^6$ infected and matured DC and $1.8 \times 10^7$ PBMC. Cultures also included 10 ng/ml each IL-7 and IL-12, added at day 0, and 10 U/ml IL2, added at day 3. Experiments for *in vitro* priming are using both CD4+ and CD8+ T cells, data is shown here for CD8. Prior to the second stimulation, $CD8^+$ cells were purified from the bulk culture using MACS columns, and CD8+ cells were restimulated in 24 well plates with Adenovirus-ICD infected DC as APC. The culture was stimulated twice more using autologous fibroblasts retrovirally transduced with ICD and tested for ICD-specific CTL activity by $^{51}$Cr-release assay. As shown in Figure 2, the bulk line contained activity specific for ICD, since the line lysed autologous B-LCL infected with vaccinia-ICD but not vaccinia-EGFP or uninfected autologous B-LCL targets.

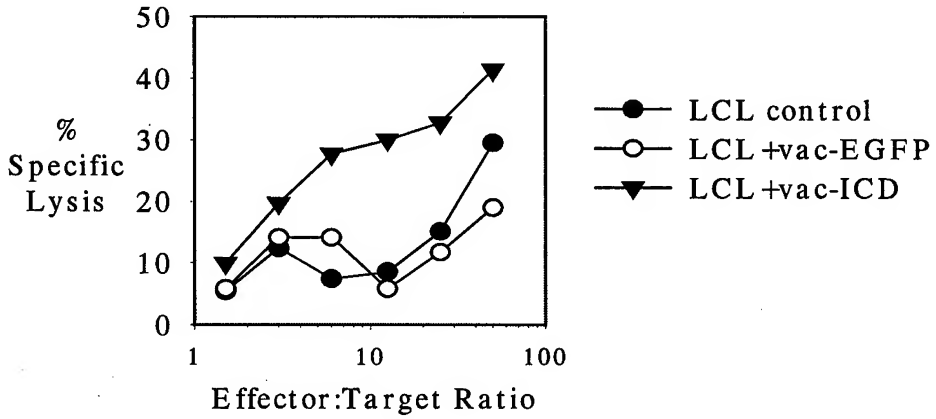
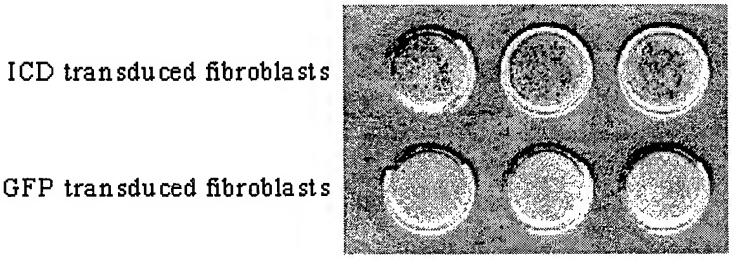



**Figure 2**: $^{51}$Cr release assay demonstrating ICD-specific CTL activity in a T cell line generated by priming with DC infected with recombinant Adenovirus-expressing ICD. Assay was a standard 4 hour $^{51}$Cr release assay; targets were autologous B-LCL infected with recombinant vaccinia expressing ICD, EGFP, or uninfected. Each data point is the average of 3 measurements.

The ICD-specific line was then re-stimulated once on autologous LCL infected with vaccinia-ICD, and then split into two and stimulated either on autologous DC infected with Adenovirus-ICD or with anti-CD3; both of these sub-lines were tested for recognition of target cells that expressed ICD using an IFNg ELISPOT assay. As shown in Figure 3, ICD-specific reactivity could be detected in both antigen (panel A) or anti-CD3 expanded (panel B) cultures. The average spot number from the triplicate wells was 344 on the ICD fibroblasts and 22 on the EGFP fibroblasts (antigen stimulation) and 365 (ICD) and 17 (EGFP) (anti-CD3 expansion).

A.

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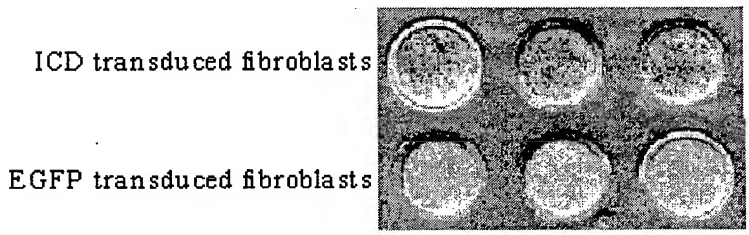


**Figure 3**: IFNg ELISPOT analysis of CD8+ T cell lines derived from in vitro priming experiments using Adenovirus-ICD infected DC as APC. Data shown are in triplicate, from two sub-lines expanded for one cycle either on Adenovirus-ICD-infected DC (A) or anti-CD3 (B), tested on autologous fibroblasts transduced with ICD or EGFP. Fibroblasts were treated with IFNg 48-72 hours prior to the assay and washed to remove cytokine. $2x10^3$ stimulators were plated per well with $2x10^4$ responders for the original cell line and $4x10^4$ responders for the expanded cell line.

An ICD-specific clone isolated from the bulk line, clone 17D5, was tested by IFNg ELIspot assay for recognition of autologous fibroblasts either untransduced or transduced with ICD, full-length HER2, or EGFP. Additionally, monoclonal antibodies that specifically blocked HLA Class I presentation were used to determine the restricting allele for the ICD response. As shown in Table 3, clone 17D5 specifically recognizes autologous fibroblasts transduced with ICD or full-length HER2, but not untransduced fibroblasts or fibroblasts transduced with the irrelevant antigen EGFP. Furthermore, this reactivity was completely blocked by the addition of a monoclonal antibody specific for HLA-B and C alleles (BB123.2), and a pan-HLA Class I monoclonal antibody (w6/32), and the reactivity was not blocked by an antibody specific for HLA-A2 (BB7.2). These results suggest that this clone is restricted by an HLA-B or C allele, the same pattern of HLA-restriction that was observed for the bulk cell line from which this clone was derived.

**Table 3**. IFNg ELIspot assay testing Her-2/neu-reactivity and HLA-restriction of the ICD specific clone 17D5.

| | **Blocking Antibody** | | | |
|---|---|---|---|---|
| **Stimulators** | none | w6/32 | BB123.2 | BB7.2 |
| Fibros | 0 | 0 | 0 | 0 |
| EGFP-Fibros | 0 | 0 | 1 | 0 |
| ICD-Fibros | 162 | 3 | 1 | 165 |
| H2N-Fibros | 104 | 0 | 0 | 98 |
| T cell alone | 0 | 0 | 0 | 0 |

New primings were established using the same donor from which the original ICD-specific T cells were derived in order to: 1) determine the reproducibility of the result, and 2) compare alternate methods of *in vitro* priming to tumor antigens. Two priming cultures were set up in parallel. The first followed the "bulk culture" protocol used to establish the original ICD-specific T cell line, with whole PBMC as responders and adenovirus-ICD-infected and matured DC as stimulators. The parallel priming used CD8+ cells as responders with the same adenovirus-ICD-infected and matured DC as stimulators. In both primings, the responder to stimulator ratio was 10:1. The cultures with CD8+ responders were set up in 96-well U-bottom plates with 15,000 DC/well and 150,000 CD8+ cells /well. IL12 and IL7 were added to both primings on day 0 at 10ng/ml each, and IL2 was added on day 3 at 10U/ml. The 96-well cultures were maintained and tested as separate "micro-cultures." IL2 was the only cytokine used in the restimulations. The same APC were used for restimulating both priming cultures. The second stimulation was on DC infected with adenovirus-ICD, and the third and

subsequent restimulations were on autologous fibroblasts transduced with ICD. Following the fourthstimulation *in vitro*, the bulk and 96-well primings were tested for specificity in a IFN ELIspot assay. The APC in the assay were autologous fibroblasts infected with adenovirus-ICD or adenovirus-EGFP at 2,000 cells per well. For the 96-well cultures, 50 ul of each 200ul culture was tested against each APC in singlicate. For the bulk culture, roughly 20,000 T cells/ well were tested in duplicate against each APC. Three of 77 micro-cultures appeared to be reactive with ICD+ fibroblasts, while the bulk culture did not appear to secrete INF in response to ICD+ fibroblasts (Table 4A).

The cell lines were tested again by IFN ELIspot assay following the fifth stimulation *in vitro*. The APC in this assay were either untransduced, autologous fibroblasts or fibroblasts transduced with ICD at 2,000 cells per well. This time 10/77 microcultures showed some reactivity with ICD+ fibroblasts. These 10 cultures included 2 of the 3 that tested positive in the first assay (A4 and F4). The potential positive cultures, as well as 2 or 3 "non-specific" negative control cultures, were kept for restimulation and expansion. The remaining micro-cultures were discarded at this time. The bulk culture showed weak reactivity with ICD+ fibroblasts in this assay (Table 4B).

No testing was done following the sixth stimulation, in order to attempt to expand the T cell lines. Following the seventh stimulation, the bulk culture was retested by ELIspot assay with a negative result (not shown). The eighth stimulation of the micro-cultures was done using anti-CD3 to expand the T cells to greater numbers. Following this stimulation, each culture was tested in duplicate by IFN ELIspot against autologous fibroblasts infected with adenovirus-EGFP or –ICD and against fibroblasts transduced with EGFP or ICD. APC were used at 5,000 cells per well, and the number of T cells per well was unknown but estimated to be between 20,000-10,000 per well. In this assay, only line "F4" secreted IFN in response to both of the ICD+ stimulator cells (Table 4C). The other microcultures and the bulk line appear to have lost reactivity for ICD, at least as detectable in this assay. This may be a result of outgrowth of non-specific cells due to non-specific expansion of the cultures on anti-CD3; however, the bulk line was not expanded on anti-CD3. It is likely that cloning positive lines at an earlier stage would be beneficial. The F4 line and the A4 line will be cloned in an attempt to isolate clones specific for ICD.

**Table 4**

A. γ **IFN Elispot following 4th stimulation in vitro**

| | STIMULATORS | |
|---|---|---|
| T cells: | AdV-EGFP | AdV-ICD |
| A4 | 5* | 100 |
| F4 | 58 | 240 |
| E11 | 12 | 25 |
| bulk line | 1 | 0 |

B. γ **IFN Elispot following 5th stimulation in vitro**

| | STIMULATORS | |
|---|---|---|
| T cells: | untransd. | ICD+ |
| A4 | 0 | 40 |
| F4 | 2 | 300 |
| E11 | 0 | 1 |
| B1 | 2 | 23 |
| B2 | 12 | 68 |
| A12 | 1 | 20 |
| B11 | 4 | 60 |
| C12 | 1 | 30 |
| E9 | 50 | >100 |
| bulk line | 2 | 32 |

C. γ **IFN Elispot following 8th stimulation in vitro**

| | STIMULATORS | | | |
|---|---|---|---|---|
| T cells: | EGFP+ | ICD+ | AdV-EGFP | AdV-ICD |
| A4 | 0 | 0 | 4 | 1 |
| F4 | 2 | 71 | 0 | 33 |
| bulk line | 42 | 25 | 32 | 28 |

*Numbers represent number of ELIspot per well in single or duplicate wells.

The conclusion thus far is that priming with Adenovirus recombinant for ICD can elicit T cells specific for ICD in a reproducible manner. Furthermore, the use of a 96-well protocol using CD8+ cells as the initial responders appears to be a more efficient and sensitive method of isolating specific T cells. This protocol will be used for attempts to prime *in vitro* to other tumor antigens. This protocol is also being applied to the generation of CD4+ T cells as well as CD8+ CTL.

Once clones are established as described above. Epitope mapping will be initiated to identify immunogenic epitopes of the HER2 protein.

Task 8: Months 13-48 To determine whether Th immunity to HER2 can be generated using T cells from individuals with HER2+ breast cancer and no detectable immunity to HER2. Data shown in previous progress reports and in the initial preliminary results presented in this proposal have demonstrated that some patients have preexistent T cell responses to HER2 protein and peptides. We have completed a study of 45 patients with HER2 overexpressing breast and ovarian cancers (5). Patients enrolled had not received immunosuppressive chemotherapy for at least 30 days (median 5 months, range 1-75 months). All patients were documented to be immune competent prior to entry by DTH testing using a skin test anergy battery (Table 5). Five of 45 patients (11%) were found to have a significant HER-2/neu specific T cell response as defined by a stimulation index $\geq 2.0$ (range 2.0-7.9). None of 8 patients who were HLA-A2 had a detectable IFNγ secreting T-cell precursor frequency to a well-defined HER-2/neu HLA-A2 T cell epitope, p369-377. These findings suggest that patients with advanced stage HER-2/neu overexpressing breast and ovarian cancer are immune-competent and can mount a T cell response to their tumor.

**Table 5.** DTH response to recall antigens in advanced stage HER2 overexpressing breast and ovarian cancer patients

| Antigen Tested | Number of Responsive Patients | Mean Response | Range of Response |
|---|---|---|---|
| Tetanus Toxoid | 28 | 8 | 3-22 |
| Diphtheria | 9 | 6 | 3-12 |
| Streptococcus | 7 | 4 | 3-6 |
| Tuberculin | 5 | 4 | 2-10 |
| Candida | 12 | 4 | 2-10 |
| Trichophyton | 6 | 3 | 2-5 |
| Proteus | 25 | 5 | 2-15 |
| Median Number of Antigens Responded to: 3 (range 2-7) | | | |

Positive Response Defined as DTH>2mm$^2$

Subsequent studies have shown that HER2-specific T cells can be generated by *in vitro* priming in patients who have some evidence of a precursor frequency prior to the initiation of the cultures. Therefore, this aim is completed. We find that one of the keys to *ex vivo* expansion of HER2 specific T cells is a detectable endogenous immunity or increase of precursor frequency post immunization.

Task 9: Months 13-48 To determine the prevalence of CD4+ responses in patients with HER2+ tumors and the evolution of immunity with therapy and relapse. Work shown in the previous report demonstrated the development of serial assays to evaluate the CD4+ cell response over time. We described a modified limiting dilution analysis which allows the identification of even low precursor frequency events (5, 6). We have now analyzed this assay for reproducibility and have begun to evaluate PBMC from patients over time (derived from the studies described above for antibody analysis). To validate the reproducibility of the assay we evaluated the CD4+ T cell response to multiple antigens in 10 volunteer donors drawn at 3 time periods over the course of 2 months. PBMC was analyzed in the modified LDA either fresh or frozen and stimulation index calculated form 24 well replicates was used for comparisons.

**Table 6.** Statistical Differences in T Cell Proliferative Assays Performed on Non-Cryopreserved Cells from the Same Donors at Three Different Time Points

| Antigen Tested | p Values Comparing Time Points | | |
|---|---|---|---|
| | Time 1 vs. 2 | Time 2 vs. 3 | Time 1 vs. 3 |
| Tetanus Toxoid | 0.754 | 0.754 | 0.754 |
| Streptokinase | 0.754 | 1.0 | 0.344 |
| Myelin Basic Protein | ND | 1.0 | 0.344 |
| PHA | 0.754 | 1.0 | 1.0 |
| CON A | 0.754 | 1.0 | 1.0 |

**Table 7.** Statistical Difference in Proliferative T cell Responses (S.I.≥2.0) Between Non-Cryopreserved (Fresh) vs. Cryopreserved (Frozen) Samples

| Antigen Tested | Number of Comparisons | p value (Fresh vs. Frozen) [a] |
|---|---|---|
| Tetanus Toxoid | 30 | <0.0001 |
| Streptokinase | 30 | 0.039 |
| Myelin Basic Protein | 30 | 1.000 |
| PHA | 30 | 1.000 |
| CON A | 30 | 1.000 |

a. Binomial distribution used

This data demonstrates that the assay is reproducible over time and analysis over time can be compared. However, cryopreserved cells lose the ability to significantly respond to antigen. Therefore, in the studies evaluating T helper responses over time, PBMC will be analyzed fresh, without cryopreservation.

Task 10: Months 13-48 To determine whether CD4+ responses modulate the biology of autologous tumors *in vivo.* We have begun to answer this question using an animal model. Initial studies have concentrated on the use of peptide vaccines targeting rat neu in the neu tg mouse. Initial results indicate a measurable CD4+ T cell response can protect an animal from the development of HER2 overexpressing breast cancer (Fig. 4).

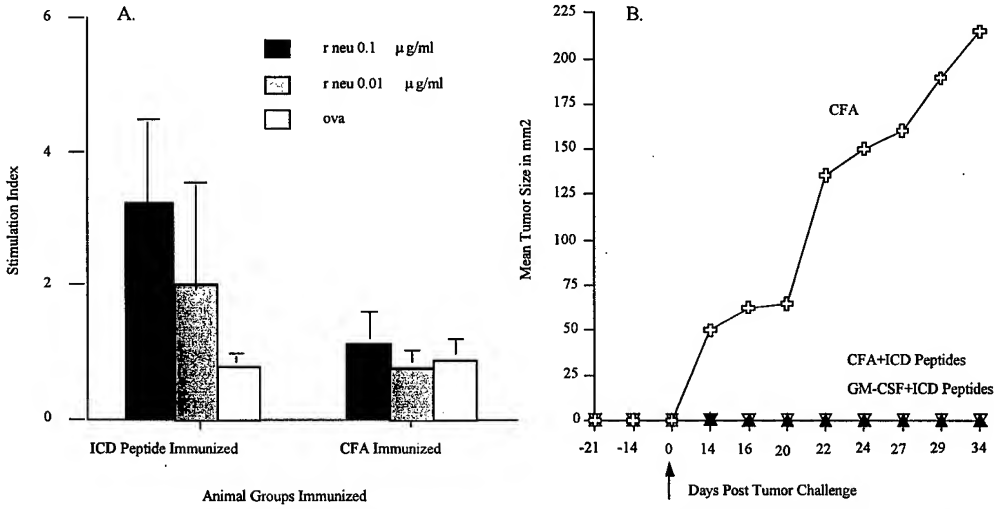


**Figure 4**: Peptide-based vaccines can protect neu-tg mice from HER2 expressing tumors. **A.** Neu-tg mice were immunized with a neu ICD peptide based vaccine at 14 day intervals for 2 inoculations. Panel A represents T cell response to neu protein at 2 concentrations as well as an irrelevant antigen, ova albumin, after peptide immunization. T cells were derived from mouse spleen. Data is expressed as the mean and standard deviation of 5 mice/group. **B.** Represents data from neu-tg mice immunized with a HER2 ICD peptide based vaccine twice at 14 day intervals prior to sq. injection of $2x10^6$ syngeneic at neu overexpressing tumor cells. Whether GM-CSF or CFA was used as an adjuvant in the initial immunizations, all animals rejected tumor (5 mice/group).

Peptide based vaccine strategies, however, might not be applicable to a widespread population, as the HLA restrictions associated with peptide immunization may be significant. Therefore, we have been exploring the use of DNA immunization as a way to generate and evaluate the HER2-specific immune response. Nucleic acid immunization, if effective, could be readily extrapolated to human clinical trials. CD4 T cell responses (proliferation and cytokine secretion) were assessed from mice immunized with naked DNA (100 ug 3x) or ICD/Montanide (50 ug 2x). Two weeks following the last immunization, spleen cells were harvested and stimulated *in vitro* with recombinant ICD or ECD protein for 4d. Supernatants were collected after 72 hrs for the measurement of IL-5 and IFN-g. 3H-thymidine incorporation was assessed after 4d. IL-5 levels were below detection in all cultures. Naked DNA induces greater cytokine release. ICD protein immunization induces greater proliferation. It appears that DNA immunization results in a stronger Th1 cytokine response than protein immunization; whereas protein immunization leads to a stronger proliferative response than DNA immunization. These observations (IFN g secretion, but little proliferation) are consistent with published reports on DNA immunizations to other Ags.

**Table 8.** CD4 T cell responses (proliferation and cytokine secretion) from mice immunized with naked DNA or ICD protein

| immunization | Mouse # | Stimulation index | | | IFNg (ng/ml) | | |
|---|---|---|---|---|---|---|---|
| | | media | 10 ug/ml ICD | 10 ug/ml ECD | media | 10 ug/ml ICD | 10 ug/ml ECD |
| VR1012-H2N | 1 | 1 | **4.6** | **2.8** | 0.42 | **8.15** | **0.42** |
| | 2 | 1 | **6.2** | **2.3** | 0.17 | **2.86** | **0.02** |
| | 3 | 1 | **3.9** | **4.0** | 0.30 | **3.17** | **2.78** |
| | 4 | 1 | **4.7** | **3.3** | 0.40 | **2.50** | **1.86** |
| | | | | | | | |
| VR1012 | 1 | 1 | 2.7 | 1.3 | 0.26 | 0.92 | 0.06 |
| | 2 | 1 | 1.0 | 0.9 | 1.51 | 1.00 | 1.08 |
| | 3 | 1 | 2.1 | 0.9 | 0.50 | 2.80 | 0.44 |
| | 4 | 1 | 1.4 | 1.3 | 0.16 | 0.72 | 0.11 |
| | | | | | | | |
| ICD/Montanide | 1 | 1 | **8.9** | 0.8 | 0.16 | 2.41 | 0.04 |
| | 2 | 1 | **10.4** | 1.1 | 0.14 | 0.65 | 0 |
| | 3 | 1 | **9.6** | 1.2 | 0.35 | 4.71 | 0 |
| | 4 | 1 | **9.6** | 1.4 | 0.57 | 0.89 | 0 |
| | | | | | | | |
| Montanide | 1 | 1 | 1.1 | 0.9 | 3.11 | 3.65 | 3.79 |
| | 2 | 1 | 1.4 | 0.8 | 1.28 | 1.90 | 0.71 |
| | 3 | 1 | 1.8 | 1.1 | 0.30 | 0.72 | 0.46 |
| | 4 | 1 | 1.5 | 0.9 | 0.50 | 1.07 | 0.45 |

**Specific Aim # 3: To examine CD8+ CTL immunity to HER2.**

Task 11: Months 1-48 To develop *in vitro* priming with dendritic APC to generate HER2 specific CD8+ T cells and to identify the epitopes recognized. Studies described above demonstrate our work with adenovirus transduced DC as an agent for *in vitro* priming. Additionally, we have found that increasing the precursor frequency of CD8+ CTL *in vivo*, by vaccination, prior to an attempt at *ex vivo* expansion will greatly facilitate the ability to grow HER2 specific T cells *in vitro*. Figure 5 demonstrates breast cancer patients immunized with HER2 HLA-A2 binding peptides do develop CTL responses that are specific for peptide and HLA A2 tumor.

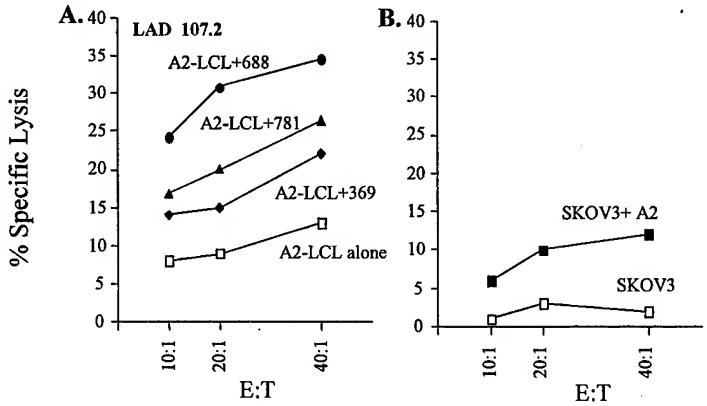


**Figure 5.** Patients immunized with HER2 peptides develop HER2 peptide and protein-specific CTL responses. Data is shown from a breast cancer patient immunized with the CTL peptide vaccine; LAD0107. Panel **A**: PBMC was taken from the patient 30 days after the second CTL immunization. $20x10^6$ cells were bulk cultured with 1.0 uM each of the HLA-A2 binding 9 mers in the immunizing vaccine. Cells were cultured for 10 days and IL-2 5U/ml was added throughout the cycle. After 1 IVS, the lines were tested for lytic activity in a 4 hour CRA against HLA-A2 LCL alone and HLA-A2 LCL coated with HER2 HLA-A2 CTL binding peptides. Panel **B**: T cell lines were also evaluated for their ability to lyse SKOV3, a tumor cell line which overexpresses the HER2 protein, and SKOV3 transfected with HLA-A2

In addition, CTL precursor frequencies can be boosted by vaccination (Fig. 6).

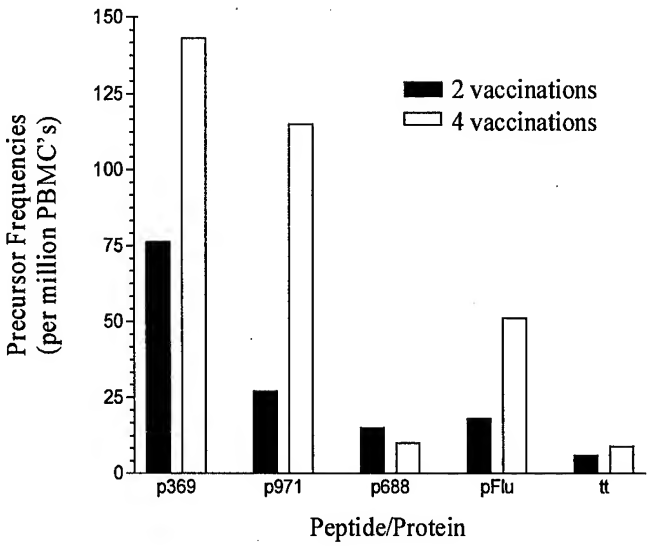


**Figure 6.** Increase in precursor frequency of IFN- secreting T cells after immunization with a HER2 peptide-based vaccine. PBMCs were isolated from a patient following the 2nd and 4th vaccinations. The PBMCs were analyzed for the presence of p369•9 peptide-specific IFN- secreting T cells using ELIspot . Results shown are the mean of 6 replicates for each determination and are expressed as the number of p369•9-specific T cells per million PBMC's.

Results in Figures 7 and 8 demonstrate that once the precursor frequency has been boosted, both the CD3 T cell number and the lytic activity of the T cells can be enhanced after only 2 IVS on antigen. Data is shown for a patient whose frequency to p369 was undetectable (<1:100,000) prior to immunization and was boosted to 1:50,000 after immunization.

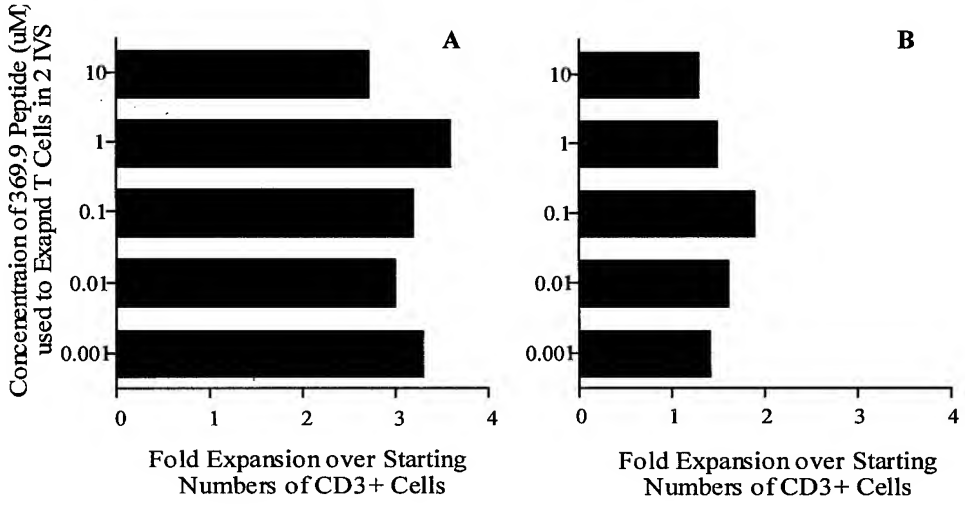


**Figure 7.** T cells can be expanded in number through 2 IVS on antigen. Data is shown from 2 patients who finished all 6 HER-2/neu peptide vaccines. Both patients were HLA-A2 and responded predominantly to p369•15, which contains a CTL epitope of HER-2/neu for HLA-A2. Bulk cultures were initiated in an identical fashion as is described in Experimental Design. The graphs here

represent the expansion of T cells (CD3+) over baseline after 2 IVS with p369.9 peptide. (**A**) depicts results from CW7561, (**B**) shows results from LR0756.

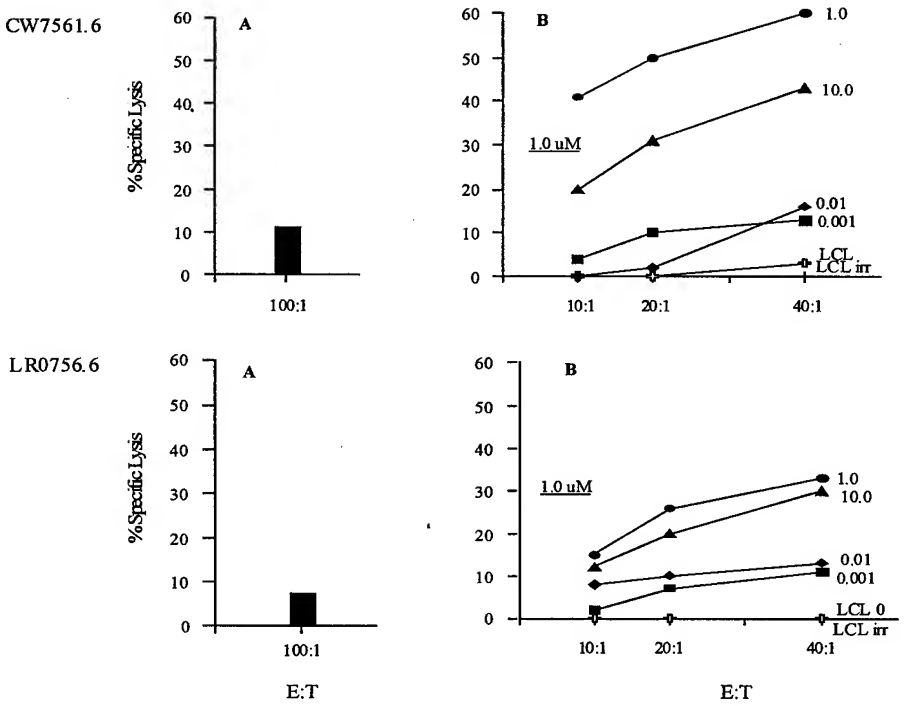


**Figure 8.** Lytic activity of HER2-specific T cells can be enriched in 2 IVS on antigen. Data is shown on two patients who finished all 6 HER-2/neu peptide vaccines. The dominant response in each patient was directed to p369•15, a 15 mer that contains an HLA-A2 CTL epitope for HER-2/neu. Both patients are HLA-A2. Panel **A** for each patient demonstrates the level of lytic activity of T cells after 1 IVS at a 100:1 E:T ratio against autologous LCL loaded with 369•9 HLA-A2 binding peptide (LCL alone lysis <1% in each case). Panel **B** for each patient represents the level of lysis against LCL coated with 369•9 HLA-A2 binding peptide after 2 IVS. Targets were coated with varying concentrations of peptides. Both cultures were established by *in vitro* stimulation with 1.0 uM immunizing peptide.

CD8 T cells will be expanded *ex vivo* based on protocols established above from patients who have been immunized and whose CTL can lyse HLA matched tumors as well as generate lines from patients with a pre-existent immune response using autologous DC.

Task 12: Months 1-36 To determine whether CD8+ CTL immunity can be generated using T cells from individuals with HER2+ breast cancer and no detectable immunity to HER2. This task is complete. As described in the section outlining the CD4+ T cell response, we will limit our studies to patients who already have a pre-existent CD8+ immune response or who have been immunized to HER2 and have a boosted precursor frequency.

Task 13: Months 37-48 To determine the prevalence of CD8+ responses in patients with HER2+ tumors and the evolution of immunity with therapy and relapse. Previous progress reports have shown that we have developed robust and reproducible assay to monitor HER2 specific CTL precursor frequency. As an example we have begun to establish baseline responses in breast cancer patients whose tumors overexpress HER2. Data shown in Table 9 is restricted to the HER2 HLA-A2 response. Work is ongoing in developing assays which will evaluate a quantitative response against HER2-transduced autologous targets such as fibroblasts and LCL.

**Table 9**. HER2 specific p369 precursor frequency on breast cancer patients with HER2 overexpressing tumors

| HER-2/neu Overexpressing HLA-A2 Patients | PHA T Cell Response (S.I.) | Frequency to Flu Matrix peptide p58-66 | Frequency to HER-2/neu peptide p369-377 |
|---|---|---|---|
| HLA A2 Control* | 72 | 1:15,700 | <1:100,000 |
| 7761 | 31 | <1:100,000 | <1:100,000 |
| 4723 | 28 | 1:16,100 | <1:100,000 |
| 6374 | 55 | nd | <1:100,000 |
| 5806 | 40 | 1:13,700 | <1:100,000 |
| 0107 | 20 | 1:23,810 | <1:100,000 |
| 2859 | 113 | <1:100,000 | <1:100,000 |
| 0756 | 103 | 1:16,700 | <1:100,000 |
| 9465 | 111 | 1:31,250 | <1:100,000 |

nd: not done

* Representative of 5 control donors evaluated

Task 14: Months 13-48 To determine whether HER2-specific CD8+ CTL derived from patients with breast cancer can lyse autologous tumor and can be expanded *in vitro* to the extent needed for adoptive therapy. We have begun to clone CTL specific for HER2 from the PBL of patients with HER2 positive tumors as described above. The generation of CD8+ HER2 specific lines and clones will allow the assessment and characterization of *in vitro* activity for potential *in vitro* correlations as well as develop the *in vitro* expansion techniques needed for expansion of HER2 specific T cells for potential use in treatment.

## KEY RESEARCH ACCOMPLISHMENTS

- Determined the prevalence of HER2 antibodies in patients with both early and advanced stage breast cancers.
- Demonstrated a significant correlation between the development of HER2-specific antibodies with HER2 overexpression in tumor.
- Defined 5 potentially novel breast cancer antigens using HER2 immune sera from breast cancer patients as a probe for E. Coli libraries (SEREX).
- Established collaborations for the collection of statistically significant numbers of sera to determine the role of HER2 antibodies in predicting prognosis, in DCIS, and as a tumor marker when followed over the course of disease.
- Established a reproducible *in vitro* priming method to expand HER2 specific T cells *ex vivo* using autologous DC.
- Established an *in vitro* culture system for the expansion of HER2 specific T cells post-vaccination.
- Determined that HER2-specific T cells can only be expanded reproducibly from patients who have a pre-existent measurable HER2-specific T cell immune response either generated endogenously or by vaccination.

## CONCLUSIONS:

The overall goal for the proposal is to develop the knowledge base necessary to develop vaccine and T cell therapy strategies directed against HER2. The studies thus far have validated that immunity to HER2 exists and can be augmented by manipulation *in vitro*. The studies have thus far resulted in a Phase I study of a HER2 peptide-based vaccine (funded by an NIH R01) and provided the foundation for the development of HER2-specific adoptive immunotherapy. We anticipate at the end of the funding of this proposal we will have initiated a protocol for the infusion of HER2-specific T cells for the treatment of advanced stage HER2 overexpressing cancers.

**Studies of antibody responses** confirmed that Ab immunity to HER2 can be detected in the sera of some patients with breast cancer and is correlated with antigen expression. The increased frequency of Ab in patients with HER2+ cancer strongly implies that immunity develops as the result of the overexpression of HER2 on breast cancer, i.e., some patients become immune to their own cancers. We now know the prevelance of HER2 antibody in patients with early and advanced stage HER2 breast cancers. In addition, we are developing strategies specifically to increase these antibody responses. HER2 is a functioning growth factor receptor. Ab to HER2 in some patients was directed against the ECD and was able to perturbate function. This remarkable finding strongly implies that the immune response to HER2 might directly alter growth characteristics and outcome in patients with breast cancer. Whether patients with HER2+ cancers and functional Ab to HER2 survive longer as a result of Ab is being pursued. These studies may eventually provide evidence of substantial and important host tumor interactions. The correlation between antibody and breast cancer shows that antibody responses have the potential to serve as tumor markers for detecting breast cancer. That hypothesis is being pursued. Also being pursued is the hypothesis that changes in level of Ab can detect early relapse. The major question of whether existent immunity to HER2 relates to improved survival is being addressed using sera collected from the NSABP adjuvant breast cancer trials. Sera is drawn at the time of diagnosis and all patients are receiving the same adjuvant chemotherapy regimen. Thus, HER2 reactivity can be analyzed as an independent variable. Finally, HER2 immune sera provides an excellent reagent to screen for other novel breast-related immunogen proteins and 5 potential new breast cancer antigens have already been identified.

**Studies of CD4+ T cell immunity** to HER2 confirmed that some patients with HER2-positive breast cancers exhibit primed CD4+ helper T cell responses to HER2. Finding existent T cell immunity is encouraging for the eventual use of T cell vaccines and T cell therapy given that HER2 is an abundant soluble protein, i.e., the extracellular domain (ECD) is shed. In animal models CD4+ T cells can be effective against abundant soluble proteins. We are testing highly sensitive and reproducible assays for evaluating CD4+ and CD8+ T cell responses to HER2. These assays are being used in the current studies to identify, quantify and follow existent immune responses to HER2. We are able to generate HER2 specific CD4+ T cells *in vitro* and appropriate collaborations have been established to determine the role CD4+ T cell immunity plays in clinical response.

**Studies of CD8+ T cell immunity** to HER2 confirmed that CD8+ CTL could be primed to HER2 peptides *in vitro* and that primed peptide specific CTL can lyse HER2 positive cancer cells. We have embarked on studies to determine the most efficient, physiologically relevant, means of generating HER2-specific CTL by priming *in vitro*. A major issue is whether HER2-specific CD8+ CTL derived from patients with breast cancer can lyse autologous tumor. We have been able to generate HER2 specific lines and clones and characterize them as to their potential function. Finally, we have begun the experiments necessary to determine whether HER2 specific T cells can be expanded and whether such cells can be expanded with maintenance of function to the extent presumed necessary for therapy.

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